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Ascorbic and Dehydroascorbic Acids Measured in Plasma Preserved with Dithiothreitol or Metaphosphoric Acid

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We describe a rapid method for accurately and precisely measuring ascorbic acid and dehydroascorbic acid in plasma. Total analysis time is <10 min, replicate analyses of a single pool provide precisions ≤2%, and values measured in supplemented samples agree with known concentrations of 4.68 and 11.83 mg/L. The stability and homogeneity of lyophilized plasma samples supplemented with ascorbic acid and dithiothreitol are documented. We also describe a procedure in which metaphosphoric acid (50 g/L) is used to prepare a reference material for the measurement of ascorbic acid and dehydroascorbic acid. The procedure for both acids consists of first measuring the native ascorbic acid, then reducing the dehydroascorbic acid, at neutral pH, with dithiothreitol, and finally measuring the total ascorbic acid; dehydroascorbic acid is then determined by difference. The metaphosphoric-acid-treated samples were stable at −70 °C, but stability decreased with temperature over the range examined, 4–50 °C.

Additional Keyphrases: *reference materials · analytical precautions*

Ascorbic acid (AA) and dehydroascorbic acid (DHAA) are normally present in both human and animal tissues and plasma.[3] Both the chemical and biochemical destruction of AA occurs solely through the pathway of AA to DHAA to diketogulonic acid—the first reaction being reversible, the second irreversible (*1*). Accurate measurement of AA and DHAA in biological samples such as plasma depends on a reliable method for stabilizing these materials in the matrix of interest and the availability of a reliable assay for each analyte. Such measurements are necessary to the design and interpretation of epidemiological studies defining the factors affecting the distribution of AA and DHAA in blood and other tissues.

Currently there are three general procedures for quantifying the sum of both analytes (total AA): (*a*) the dinitrophenylhydrazine method (*2*), (*b*) the *o*-phenylenediamine method (*3–6*), and (*c*) the reduction of DHAA by sulfhydryl reagents (*7–13*).

The first two procedures, both based on the production of a derivative of DHAA with either dinitrophenylhydrazine or *o*-phenylenediamine, are used to measure the DHAA content and, indirectly, to measure total AA after the native AA has been oxidized to DHAA (*1*). However, both of these reagents react with other reductones, including diketogulonic acid, and this can lead to measurement errors in a simple chromogenic assay (*1*).

The last two procedures have been used in conjunction with chromatographic methods. Method *b* has been used for both pre- and postcolumn derivatization of DHAA with *o*-phenylenediamine and method *c* for precolumn reduction of DHAA. The advantage of method *b* is that it measures DHAA directly. These last two procedures involve either complex extractions that require the use of internal standards (*4*), an insensitive ultraviolet method for AA detection (*5, 13*), or a lengthy separation procedure (*6*).

The procedure we used in this study (a variant of procedure *c*) involves sulfhydryl reagents (*7–13*) to reduce DHAA to AA, and requires little additional sample manipulation. Diketogulonic acid is unaffected by dithiothreitol (DTT). This procedure measures only AA directly; DHAA is measured indirectly after its reduction to AA. The complete assay can be done by HPLC in 8 min with use of a very sensitive electrochemical (EC) detector at a constant electrode potential. To measure DHAA, one must analyze each sample twice, once for native AA concentration and once after the addition of DTT, to measure total AA content. The DHAA content is calculated as the difference between the values for native AA and total AA.

Accurate results entail both accurate analyses and stable samples. However, there is a paucity of published data on the long-term stability of AA in acid-stabilized serum or plasma (*3–5, 14, 15*). In one report (*5*) it was demonstrated that AA and DHAA were stable when stored at −20 °C for 30 days in 100 mL/L metaphosphoric acid (MPA). In a second (*15*), AA was found to be stable for five weeks at −70 °C in 50 mL/L MPA. In two studies (*3, 14*) total AA was measured in plasma. Bradley et al. (*14*) demonstrated that AA was stable in plasma for 21 days at 4, −20, and −70 °C, in the presence of either MPA or trichloroacetic acid; Baker et al. (*3*) found AA to be stable for seven days at −70 °C in EDTA–perchloric acid. Using a colorimetric assay, they (*3, 14*) observed an initial, unexplained 5% to 13% increase in the concentration of AA within the first week of storage, and Baker et al. (*3*) noted that their colorimetric results did not agree with results they obtained with an HPLC-EC method. Nevertheless, the concentration of AA determined by the HPLC method corresponded to the amount added to AA-free plasma, suggesting a systematic bias in the determination of AA by the dinitrophenylhydrazine method. We have reported that AA in plasma can be preserved by adding DTT and have demonstrated that all of the added AA was accounted for after 88 weeks in samples stored at −70 °C (*13*).

The objectives of the present study were (*a*) to demonstrate the accuracy and precision (including some of the sources of systematic error) of the measurements determined by a modified version of our recently reported HPLC-EC method (*13*); (*b*) to demonstrate the feasibility of preparing a plasma pool suitable for use as a reference

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[3] Nonstandard abbreviations: AA, ascorbic acid; DHAA, dehydroascorbic acid; MPA, metaphosphoric acid; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; and EC, electrochemical.

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material in field studies; (*c*) to show that DHAA formed by the oxidation of AA in plasma at 22 °C can be quantitatively converted to AA; and (*d*) to demonstrate that no measurable degradation of AA occurs in MPA-treated plasma at −70 °C, but at 4, 25, and 50 °C AA is more or less slowly oxidized to DHAA and other products.

Materials and Methods[4]

Materials

AA and DTT were purchased from Sigma Chemical Co., St. Louis, MO; metaphosphoric acid from Fisher Scientific, Fair Lawn, NJ; and the plasma from Western States Plasma Corp., Fallbrook, CA. The acetonitrile was "HPLC" grade. The AA, examined by proton magnetic resonance, contained <0.1% protonated impurities, including DHAA and its degradation products. The processed plasma was stored at −20 °C until used.

The method of AA analysis is a modification of that described in reference *13* as HPLC Method 1. A 100 × 7.8 mm "fast acid analysis" column (Bio-Rad Laboratories, Richmond, CA) was used at a flow rate of 1 mL/min and the EC detector was a Model 400 (EG&G, Princeton Applied Research, Princeton, NJ). Analysis time was shortened from 35 min to 8 min. A series of three calibration standards (three AA concentrations that bracketed the sample concentrations) were run at the beginning and end of each day, to assess the stability of the detector and to provide a calibration curve for use in calculating the AA concentration of the samples (*13*). All serum samples were aliquoted with a calibrated automated positive-displacement pipet in either the dispensing (for reference-material preparation) or diluting (for sample analysis) mode.

Preparation of AA-Supplemented Plasma Samples

Set 1. Quality-control sample set. We prepared three 160-sample groups of lyophilized plasma: (*a*) a group of unsupplemented plasma samples, (*b*) a group supplemented to give a low-normal concentration of AA, and (*c*) a group supplemented to give a high-normal concentration of AA. DTT was added to plasma at room temperature to give a concentration of 1 g/L. This plasma (relative density 1.025) was divided into three lots, two of which were supplemented by adding a weighed amount of an AA solution (1.184 g of AA per gram of 10 g/L aqueous DTT:acetonitrile, 1:3 by vol), such that the final AA concentration of each lot of reference material was 4.68 and 11.83 mg per liter of plasma for the low- and high-normal samples, respectively. Each lot of reference material was well mixed, after which 1-mL samples were aliquoted, lyophilized, and stored at −70 °C as previously described (*13*).

Each sample was prepared for analysis by equilibrating it at room temperature and adding 0.97 mL of distilled water. After the solids were completely dissolved, 0.1 mL of reconstituted plasma was diluted with 1 mL of distilled water into a vial containing 160 μL of a 400 g/L solution of metaphosphoric acid; 400 μL of acetonitrile was added, and the vial was capped and vortex-mixed for 15 s. The resulting suspension was centrifuged (1000 × *g*, 30 min, 4 °C) and the supernatant fluid was transferred to 1.8-mL autosampler vials and analyzed by HPLC.

Set 2. Samples preserved with metaphosphoric acid. The frozen human plasma was thawed and three 100-sample lots were prepared: (*a*) a group of unsupplemented plasma samples, (*b*) a group supplemented with a low-normal concentration of AA, and (*c*) a group supplemented with a high-normal concentration of AA. Two lots of plasma were supplemented by adding a weighed amount of an AA solution (49.56 mg of AA per 53.24 g of aqueous metaphosphoric acid, 100 g/L) such that the final concentration of AA in each lot was 4.87 and 11.75 mg per liter of plasma (low and high normal, respectively). Each plasma lot was thoroughly mixed. The solutions were exposed to 22 °C for 2 h, permitting partial oxidation of AA to DHAA. Therefore, the *b* and *c* samples could be used to analyze for both AA and DHAA, and they mimicked real samples. Of these plasma samples, 0.5-mL aliquots were then mixed with 0.5 mL of a 100 g/L solution of metaphosphoric acid, in glass ampules, which were then sealed, their contents frozen, and stored at −70 °C.

At the time of analysis, the test-plasma samples preserved with metaphosphoric acid were thawed in a water bath at 20 °C; thawing was complete within 10 min. Two analyses were performed on each sample, the first to measure the native AA content and the second to measure the total AA (i.e., AA + DHAA) after reduction with DTT.

Samples for the first analysis were prepared by diluting 0.200 mL of sample with 1 mL of distilled water in a vial containing 160 μL of metaphosphoric acid (400 g/L); 0.400 mL of acetonitrile was added, the vial was capped, and the sample was vortex-mixed for 15 s. After centrifuging the resulting suspension (1000 × *g*, 30 min, 4 °C), we transferred the supernatant fluid to 1.8-mL autosampler vials and analyzed it by HPLC.

The samples for the second analysis were prepared in the same manner as for the first analysis except that 200 μL of test plasma sample was diluted with 1 mL of distilled water in a vial containing 200 μL of a solution of 0.5 mol of K_2HPO_4 and 1 g of DTT per liter. After 1 h at room temperature, 160 μL of a 400 g/L MPA solution and 400 μL of acetonitrile were added, and samples were vortex-mixed and centrifuged as above. The supernatant fluid was then transferred to autosampler vials for analysis. All HPLC-EC measurements were corrected by calibration factors.

Statistical analysis. Measurement data on the samples from set 1 were examined. Separate analyses of variance were made for the low- and the high-concentration plasma samples. The organization of the data was that of a four-level hierarchical design. Analyses were made over 12 different days, with two different samples for each day, two aliquots taken from each sample, and two measurements made on each aliquot. Thus, for each concentration, 96 measurement results were used and four sources of variance were evaluated: days, samples, aliquots, and replicate measurements.

Results

The chromatograms of the unsupplemented plasma (Figure 1, *A* and *B*) indicate that it contained no electrochemically active material with a retention time at or near that of AA. Furthermore, treatment of this plasma with DTT did not result in the generation of any material that

[4] Identification of any commercial product does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

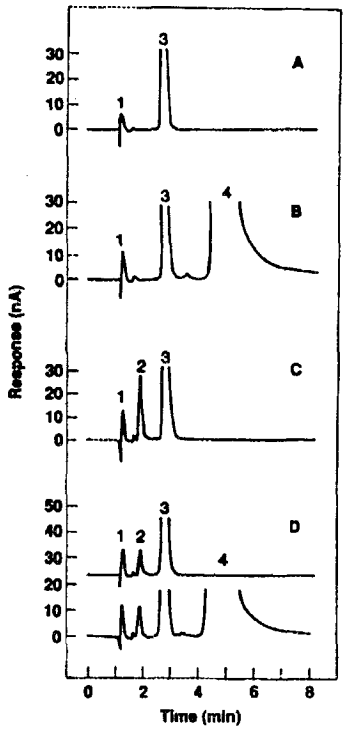

Fig. 1. Chromatograms of lyophilized and MPA-preserved human plasma and AA-supplemented plasma with and without DTT treatment

A, lyophilized plasma; *B*, plasma preserved in MPA (50 g/L) and treated with dipotassium phosphate (0.05 mol/L) and DTT (1 g/L) for 1 h; *C*, lyophilized plasma containing DTT (1 g/L) and supplemented with 11.83 mg of AA per liter; *D*, plasma preserved with MPA (50 g/L) and supplemented with AA, 11.75 mg/L, *upper trace*, no DTT, *lower trace*, treated with DTT (1 g/L) and dipotassium phosphate (0.05 mol/L) for 1 h. Peak *1*, MPA; *2*, AA; *3*, uric acid; *4*, DTT

co-chromatographed with AA. Thus the unsupplemented plasma matrix contained neither AA nor DHAA (which was reduced to AA with DTT), and all of the material measured in all samples represented either added AA or material converted to AA.

Measurement of AA in Lyophilized Plasma Stored at −70 °C (Set 1)

The concentration of AA in the two supplemented lots of plasma (AA, 4.68 and 11.83 mg/L) was measured in duplicate on each of 12 days over a 21-day period. Twenty-four samples were selected without conscious bias from each lot and two were analyzed on each day. Analysis of variance of these data (Table 1) gives the various sources of imprecision contributing to the total variability of a single measurement. The total imprecision (i.e., the CV) of a single measurement for each lot of samples was 2% (11.76 ± 0.26 and 4.70 ± 0.11 mg/L). This uncertainty is small and the measured values vary from the gravimetric value by <1 SD (Table 1). These results demonstrate the stability and homogeneity of these reference materials relative to the day of analysis and the order of sample preparation.

Measurement of AA and DHAA in Frozen Plasma Preserved with MPA (Set 2)

The following studies were done to examine the accuracy and precision of the measurement of existing AA, total AA after reduction of the DHAA in the serum sample with DTT, and DHAA by difference (total AA − existing AA). Because the reduction of DHAA by DTT is pH dependent (7), we adjusted the pH of each sample with dipotassium phosphate. The precipitated material completely dissolved and the DHAA was completely reduced to AA within 10 min at room temperature (22 °C). The resulting AA concentration remained constant for at least 90 min after the sample was prepared. In the absence of the dipotassium phosphate the reduction of the DHAA was still incomplete after the mixture had stood for an hour at room temperature.

Table 1. Summary of Analysis of Variance of Plasma Samples, Set 1, Supplemented with AA at High- and Low-Normal Concentrations

	Ascorbic acid, mg/L	
AA added	11.83	4.68
Grand mean of all 96 measurements	11.76	4.70
Source of variability	Component of SD, mg/L	
Day	ns[a]	0.07
Sample	0.23	ns[a]
Aliquot	0.07	0.06
Replicate measurement	0.10	0.06
SD of a single measurement	0.26	0.11
CV, %	~2	~2

[a] ns = not significant.

Before being preserved with MPA, the initial decrease in AA (11.75 and 4.87 mg/L) in plasma at 22 °C for 2 h was 1.60 and 0.98 mg/L, respectively (Table 2). After being preserved with MPA, these samples were analyzed for DHAA content and for analytical recovery of supplemented AA (i.e., AA + DHAA content) over a period of 98 days. The total AA remained constant in MPA (50 g/L) when the sample was stored at −70 °C. These results indicate that the oxidized AA, namely DHAA, could be quantitatively converted to AA by DTT, and that no diketogulonic acid was formed. This is demonstrated by the fact that the values obtained for AA after DTT treatment (11.69 and 5.03 mg/L) were not statistically different ($P > 0.05$, Student's *t*-test) from the amount gravimetrically added to the plasma at the beginning of the experiment (11.75 and 4.87 mg/L, Table 2). These results demonstrate the relative homogeneity of the samples preserved with MPA and indicate that the sample-to-sample variation in total AA

Table 2. Summary of Analyses of AA-Supplemented Plasma Samples, Set 2, Preserved in MPA, 50 g/L

	Concentration, mg/L	
AA added	11.75	4.87
(No. of samples)	(10)	(5)
AA[a]	10.09 ± 0.04	4.05 ± 0.13
Total AA[b]	11.69 ± 0.06	5.03 ± 0.06[c]
DHAA[d]	1.60 ± 0.07	0.98 ± 0.14

[a] Mean ± SD of the mean.

[b] Total ascorbic acid (mean ± SD of mean) was determined as described in the text with use of DTT as a reducing agent.

[c] This value does not differ significantly ($P > 0.05$ by Student's *t*-test) from the gravimetric value.

[d] Determined by subtracting the mean AA value from the mean total AA value.

content is similar to that observed for those samples prepared in DTT and then lyophilized and stored at −70 °C.

The CV for the measurement of AA in both sample lots before the reduction of DHAA with DTT, 0.4% and 3% (10.09 ± 0.04 and 4.05 ± 0.13 mg/L), is similar to the CV for the total AA value, 0.5% and 1% (11.69 ± 0.06 and 5.03 ± 0.06 mg/L, Table 2). The DHAA content (1.60 ± 0.07 and 0.98 ± 0.14 mg/L)—i.e., the "total AA" minus "AA"—can be determined to the same general precision as the "total AA" and "AA." The results indicate that <1 mg of DHAA per liter can be precisely measured.

Stability of AA in Plasma Stabilized with MPA (Set 2) at 4, 25, and 50 °C

In preliminary studies we thawed our samples by putting them in "warm water" for a variable period of time. Under these uncontrolled conditions we observed measurable variation in the initial AA content. Thus at increased temperatures, plasma AA preserved with MPA does not appear to be stable. We have now examined this instability at 4, 25, and 50 °C, using the plasma samples from set 2. At 4 °C AA degraded slowly. The degradation was detectable in the high-normal plasma samples after only one day, and it continued for 14 days (Table 3, experiments 1 and 2). In the low-normal plasma samples (Table 3, experiment 3) about 80% of the AA was lost after 14 days. More importantly, the total AA content was reduced 42%. This indicates that some of the DHAA was also degraded along with the AA. At 25 °C, detectable oxidation of AA occurred within 5 h, and after 24 h 18% of the AA had been oxidized (Table 4). At 50 °C the degradation of AA was even faster, 16% in 3 h (Table 4).

Table 3. Stability of Ascorbic and Dehydroascorbic Acids in MPA, 50 g/L, at 4 °C

	Concentration, mg/L[a]		
Day	AA	Total AA[b]	DHAA[c]
Experiment 1			
0	9.9 ± 0.22	11.6 ± 0.08	1.7
1	9.2 ± 0.19	11.6 ± 0.15	2.4
2	9.1 ± 0.10	11.0 ± 0.12	1.9
4	8.6 ± 0.12	11.2 ± 0.06	2.6
7	8.5 ± 0.09	10.6 ± 0.11	2.1
9	7.6 ± 0.06	9.9 ± 0.03	2.3
Total loss	2.3	1.7	
Experiment 2			
0	10.1 ± 0.14	11.6 ± 0.06	1.5
3	8.8 ± 0.14	10.9 ± 0.12	2.1
7	8.7 ± 0.08	11.2 ± 0.05	2.5
10	7.9 ± 0.13	10.1 ± 0.05	2.2
14	7.2 ± 0.10	9.7 ± 0.12	2.5
Total loss	2.9	1.9	
Experiment 3			
0	3.9 ± 0.18	5.0 ± 0.06	1.1
3	3.3 ± 0.10	4.6 ± 0.02	1.3
7	2.9 ± 0.07	4.4 ± 0.04	1.5
10	1.9 ± 0.10	3.6 ± 0.07	1.7
14	0.7 ± 0.06	2.9 ± 0.09	2.2
Total loss	3.2	2.1	

[a] Each number represents the mean (and SD) of four measurements.
[b] Ascorbic acid content measured after treatment of the sample with DTT.
[c] The difference between the AA value and the total AA value.

Discussion

Validity of AA and DHAA measurement in clinical plasma samples depends on the availability of a method of demonstrated precision and accuracy for measurement of each analyte. The modified method described here is characterized by increased speed of analysis (8 min), baseline peak resolution comparable with that of our previous method (*13*), and the elimination of baseline drift. Figure 1 clearly demonstrates the baseline resolution of all peaks. Furthermore, the chromatograms of the unsupplemented plasma to which DTT was added indicate that there was no AA, DHAA, or any "contaminating" peaks. However, for patients receiving special therapy, it may be necessary to demonstrate that no interfering materials are present by measuring the AA content in the presence and absence of ascorbate oxidase (EC 1.10.3.3). The baseline drift observed in earlier experiments (*13*) was reduced by use of the EC detector such that negligible signal degeneration was observed during an 8-h period.

A major source of measurement error is imprecision in sampling the plasma. This is a function of the viscosity of the plasma. We have minimized this error by using an automated diluting pipet. With this instrument, an accurate aliquot of plasma is taken and then the sampling pipet is purged with at least five volumes of diluent to wash out the viscous plasma. We tested the dependability of this method by using a single lot of stable reference plasma samples that had been supplemented with AA and preserved as previously described (*13*). The results of such an experiment (Table 1) indicate that this method accurately measures the supplemented concentrations of AA, i.e., measured values of 11.76 ± 0.26 and 4.70 ± 0.11 mg/L as compared with the respective gravimetric values of 11.83

Table 4. Stability of Ascorbic and Dehydroascorbic Acids in MPA, 50 g/L

	AA	Total AA[b]	DHAA[c]
Minutes[a]		mg/L	
Temperature 25 °C			
15	10.3[d]	12.0[d]	1.7
60	10.1	12.0	1.9
120	10.2	11.8	1.6
180	9.8	11.8	2.0
300	9.7	11.6	1.9
1440	8.4	11.0	2.6
Total loss	1.9	1.0	
Temperature 50 °C			
0	10.1	11.6	1.5
20	9.8	11.7	1.9
40	9.8	11.8	2.0
60	9.4	11.8	2.4
120	8.9	11.2	2.3
180	8.5	10.7	2.2
Total loss	1.6	0.9	

[a] Elapsed time after the sample was transferred from −70 °C directly into the controlled-temperature bath.
[b] Ascorbic acid content measured after treatment of the sample with DTT.
[c] The difference between the AA value and the total AA value.
[d] Each value represents the mean of two determinations.

and 4.68 mg/L. The precision of both the low- and high-concentration AA measurements is ~2%. The error contributions of the different components of the analytical method were assessed by analysis of variance. These components (Table 1) included (*a*) the variability due to the handling and processing of samples on different days; (*b*) the between-sample variability for a given analyte concentration; (*c*) the variability in the sample preparation procedure, including aliquoting the sample, adding reagents, and precipitating the proteins; and (*d*) variability between duplicate analyses on the same aliquot that is attributable to the variation in instrument settings. From a practical viewpoint, all four sources of variability are small. The difference between the gravimetric measurement and the observed values (an index of the accuracy of the measurement) falls within the precision of the measurement, the CV being about 2% of the measured value for a single measurement. This supports the conclusion that there is no outstanding source of systematic bias.

Furthermore these results demonstrate an acceptable level of stability and homogeneity for both sets of samples (sets 1 and 2) and clearly indicate that our measurements are reproducible for both sets of samples. These results are consistent with our previous observation (*13*) on the stability of samples that were similar to set 1. Because these samples are stable, this product may be a suitable control material for AA measurement by methods used to resolve AA and DTT.

The suitability of a method for preserving AA in biological samples collected for epidemiological studies and the valid interpretation of data from completed epidemiological studies depend on demonstrating the stability of plasma AA preserved by that method. One approach to evaluating the stability of AA in plasma is to measure, specifically, AA and its immediate degradation product, DHAA, so that storage procedures can be effectively evaluated. This is particularly important because some clinical samples may contain significant concentrations of DHAA (*5, 6*).

For us to evaluate the stability of AA and DHAA in plasma preserved with MPA, it was necessary to prepare a reference material in which the total AA (AA + DHAA) was accurately measured. This was achieved by gravimetrically adding a known amount of AA to plasma, and permitting partial oxidation of the AA to DHAA by letting the supplemented plasma stand at room temperature for 2 h. The DHAA produced this way seemed to us to most closely approach the conditions under which DHAA is generated when samples are processed in the field and preserved with MPA.

DHAA was measured after neutralizing the plasma sample and reducing the DHAA to AA with DTT. The reduction of the DHAA was complete within 10 min and no further change was observed during the following 80 min. The nonsupplemented plasma pool contained neither AA nor DHAA (Figure 1). The results in Table 1 clearly demonstrate that all of the added AA was accounted for as AA or DHAA and that both AA and total AA (and by inference the DHAA) are stable at −70 °C when preserved in MPA (50 g/L). The small standard deviation indicates that the means for both supplemented lots are precise and that all of the values from day 6 to day 98 fell within a narrow range. These results, i.e., recovery of all supplemented AA, also indicate that the use of DTT (1 g/L) in the presence of approximately 0.05 mol of dipotassium phosphate per liter (final concentration) effectively reduces all of the oxidized AA to AA. Thus, this method provides a suitable technique for measuring the sum of AA plus DHAA in biological samples after they have been preserved with MPA (50 g/L).

The identification of the degraded AA as DHAA is based on its reductive conversion to a material chromatographically identical to AA. Because the unsupplemented plasma does not contain any material that can be converted to a material that is chromatographically identical to AA or is electrochemically reducible, the material that was detected by reduction with DTT had to arise from the supplemented AA. The observation that DHAA in plasma can be reduced to AA by DTT (*13*) also supports the identification of the degraded AA as DHAA.

The studies described above (as opposed to those done on serum samples collected and preserved in field studies) were carried out under ideal conditions, where the samples were rapidly aliquoted and quickly cooled to −70 °C. At the time of assay they were carefully thawed and prepared for assay. Close attention must be paid to this procedure because of the temperature instability of AA and DHAA. A study of the stability of AA and DHAA at 4, 25, and 50 °C, in the supplemented plasma samples preserved in MPA, indicates that degradation of AA can be detected at these temperatures (Tables 3 and 4). At 4 °C, degradation of AA can be detected between 24 and 48 h. By 14 days, 29% (2.9 mg/L) of the remaining AA at the high concentration and 82% (3.2 mg/L) at the low concentration were oxidized. At 25 °C, similar results were observed within 24 h (1.9 mg/L) and at 50 °C, within 3 h (1.6 mg/L). Furthermore, at all three temperatures a measurable amount of the total AA was not accounted for at the end of the experiment; evidently some of the DHAA was further degraded. Thus, under field conditions, MPA-treated clinical samples can be stored for a day at 4 °C without significant loss of AA or of its initial degradation product (DHAA), but higher temperatures incur a risk of significant loss. Frozen samples should be thawed before analysis by warming the samples at 20 °C for 10 min to ensure minimal degradation. Furthermore, when MPA-preserved samples are kept at room temperature for extended periods in the absence of DTT, the AA can be expected to undergo degradation. These samples will not yield accurate measurements of native AA.

Under the conditions described above, we did not observe AA concentrations significantly exceeding the amount to which the plasma was supplemented. This suggests that earlier results obtained with a colorimetric method are in error, the source of which remains undefined (*3, 14*). We were also able to account for all of the DHAA that we generated. This is in contrast to the lesser recovery reported by Baker et al. (*3*), a discrepancy that may be the result of incomplete conversion of the DHAA dimer to the monomer in the plasma samples supplemented with commercial DHAA. Finally, we have confirmed that aged plasma can slowly oxidize AA to DHAA at a rate similar to that reported by Baker et al. (*3*) and that the DHAA resulting from the oxidation of AA can be determined by the method described herein.

In conclusion we have described a technique that is suitable for use in the future evaluation of the stability of AA and DHAA in whole blood and for the elucidation of suitable conditions for conserving the physiological ratio of these compounds so that the epidemiological significance of

the natural concentrations of AA and DHAA can be explored.

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